invitrogen™

SPOT-Light® HER2 CISH Kit

IVD

REF Cat. # 84-0150

Σ 20 tests using 24 mm x 30 mm coverslips

Intended use

IVD

For *in vitro* diagnostic use

The SPOT-Light® HER2 CISH Kit is intended to quantitatively determine HER2 gene amplification in formalin-fixed, paraffin-embedded (FFPE) breast carcinoma tissue sections using chromogenic in situ hybridization (CISH) and brightfield microscopy. This test should be performed in a histopathology laboratory.

The SPOT-Light® HER2 CISH Kit is indicated as an aid in the assessment of patients for whom Herceptin® (trastuzumab) treatment is being considered. The assay results are intended for use as an adjunct to the clinicopathological information currently being used as part of the management of breast cancer patients. Interpretation of test results must be made within the context of the patient's clinical history by a qualified pathologist.

Summary and explanation

The human gene HER2, or c-*erb*B-2, and the rat equivalent gene, *neu*, have been identified as proto-oncogenes[1-3] sharing homology with the closely related v-*erb*B oncogene.[1] The HER2 gene is located on chromosome 17 (17q11.2-21) and encodes a 185 kDa membrane receptor-like protein. The HER2 protein has tyrosine kinase activity and shares homology with, but is distinct from, the epidermal growth factor receptor (known as EGFr, HER1, or c-*erb*B-1).[2]

HER2 gene amplification or HER2 protein overexpression has been identified in 18–30% of human breast cancers.[8-9] Identification of HER2 gene amplification status is important for determining prognosis of patients diagnosed with invasive breast cancer, as well as in selecting patients eligible for therapy with trastuzumab (Herceptin®, F. Hoffmann-La Roche Ltd, Basel, Switzerland and Genentech, Inc., South San Francisco, CA).[4-6] Trastuzumab is a monoclonal antibody specific for the HER2 protein. Trastuzumab has been shown to be an effective therapy only in patients whose tumors show HER2 gene amplification and/or HER2 protein overexpression.[7,11] In early breast cancer, a short course of trastuzumab administered concomitantly with docetaxel or vinorelbine was shown to be effective in women with breast cancer who have an amplified *HER2/neu* gene.[12] Other studies have also shown that HER2 status predicted the sensitivity or resistance to certain chemotherapy regimens.[13] Therefore, accurate, consistent, and straightforward methods for evaluation of HER2 gene and HER2 protein status have become increasingly important.

Principles of procedure

The CISH technique uses digoxigenin-labeled DNA probes that are specific for the HER2 gene locus on chromosome 17q11.2-21 to hybridize to the complementary nucleic acids present in the breast cancer specimen. The labeled HER2 probe is made using Subtraction Probe Technology (SPT™), a proprietary and patented technology that creates specific probes by significantly reducing the repetitive sequences (e.g., Alu and LINE elements) found in human nucleic acids. The probe has been demonstrated to contain the HER2 gene by PCR, and to bind specifically to the HER2 gene locus on chromosome 17q11.2-21 by metaphase FISH in normal lymphocytes. Consequently, Invitrogen's SPT™ probes are inherently specific and do not require repetitive sequence blocking, as

REAGENTS/MATERIALS & EQUIPMENT REQUIRED BUT NOT PROVIDED

Ancillary reagents/materials

	Invitrogen Cat. no.
1. SuperFrost Plus slides **OR** Histogrip™ Slide Adhesive	00-8050
2. Positive tissue control: FFPE breast carcinoma (ductal, tubular, lobular or mixed type) tissue section with *HER2* gene amplification previously confirmed with CISH or FISH	
3. Negative tissue control: FFPE breast carcinoma (ductal, tubular, lobular or mixed type) tissue section with *HER2* gene non-amplification previously confirmed with CISH or FISH	
4. Deionized or distilled water (dH_2O)	
5. Xylene	
6. 70%, 85%, 95%, and 100% ethanol (EtOH)	
7. Coverslips, rubber cement, 18G ½" needle and 5 ml syringe **OR**	
8. UnderCover™ Slips (18 x 18 mm)	00-8403
9. UnderCover™ Slips (22 x 22 mm)	00-8404
10. 30% hydrogen peroxide (H_2O_2)	
11. 100% methanol	

Equipment

1. Timer
2. Pipette (20 µl, 1000 µl)
3. Pipette tips
4. Slide rack
5. Hot plate, aluminum foil, and 1 L beaker
6. Slide warmer
7. 37°C incubator
8. PCR thermal cycler with a slide block **OR**
   Heating block with digital thermometer and 37°C incubator (± 1°C) and humidity slide box
9. Water bath (capable of maintaining 70–80°C temperature range) with a calibrated thermometer
10. Coplin jars and staining jars
11. Brightfield microscope with 20X and 40X objectives

Precautions

- For *in vitro* diagnostic use.
- For trained professional users.
- **Do not** use kit after the expiration date printed on the label.
- If product is stored under conditions other than those specified in the package insert, then those storage conditions must be verified by the user.
- Do not eat, drink smoke, or apply cosmetics where the kit materials are being handled. Universal Good Laboratory Practices should be observed.
- No available test method can offer complete assurance of eliminating potential biohazardous risk. Handle all materials at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes for Health manual. "Biosafety in Microbiological and Biomedical Laboratories," 4th Edition, April 1999.
- Do not pipette reagents by mouth and avoid contacting skin and mucous membranes with specimens and reagents. If reagents come into contact with skin or mucous membranes, rinse affected areas with copious amounts of water.
- Reagents B, F, & G contain 0.1% sodium azide, Reagent H contains 0.005% gentamycin sulfate and 0.1% Proclin, Reagent I2 contains 85% w/v methanol, and Reagent I3 contains 0.6% hydrogen peroxide. At product concentrations, these reagents do not require hazard labeling. Please refer to the component specific MSDS for further information
- Reagent B contains pepsin, and this enzyme may cause allergic reactions in contact with the skin.

- Use temperature calibrated waterbath, heating block and hybridization oven for optimal results.
- Some of the reagents in this kit contain sodium azide as a preservative. Sodium azide may react with lead or copper plumbing to form explosive metal azides, especially if accumulated. When disposing of reagents with sodium azide, flush with large amounts of water to avoid buildup of chemical hazards in the plumbing.
- Some reagents in this kit contain Proclin, sodium azide, or hydrogen peroxide. These reagents are classified as harmful, as they are irritants to skin and mucous membranes. These substances are supplied in diluted form as components of this kit, which therefore may minimize exposure risks significantly but not completely. Avoid contact with skin, eyes, and clothing. Please refer to the component-specific MSDS for further information.
- 3,3'-diaminobenzidine tetrachloride (DAB) may be harmful if swallowed, inhaled, or absorbed through the skin, and may be irritating to eyes, skin, mucous membranes and the upper respiratory tract. DAB is a suspected carcinogen; consult Federal, State, and/or local regulations for disposal recommendations.
- Avoid evaporation of Reagent I2. Methanol evaporates easily. Take steps to avoid evaporation such as sealing the container and closing the cover immediately after use. The evaporation of methanol may cause precipitation of DAB, which may affect staining results.
- Avoid evaporation during hybridization by ensuring that the slides are properly sealed and that there is adequate humidity in the hybridization chamber.
- Enzyme pretreatment may vary depending on fixation and tissue thickness. For most tissue sections (4–5 µm) fixed with 10% neutral buffered formalin, 5 minutes of enzyme pretreatment is optimal. For tissues with unknown fixation, an enzyme titration (e.g. 2 min., 5 min., & 10 min.) should be performed with optimal digestion time adjusted based on the initial results.
- **Reagent C** – Labeled HER2 probe contains formamide which is harmful.
  R21 – Harmful in contact with skin
  R22 – Harmful if swallowed
  R61 – May cause harm to the unborn child
  S24 – Avoid contact with skin
  S36 – Wear suitable protective clothes
  S37 – Wear suitable gloves
  S39 – Wear eye/face protection
- **Reagent I2** – DAB solution contains methanol, which is flammable and toxic. Please refer to the MSDS for further information.
  R10 – Flammable
  R23/24/25 – Toxic by inhalation, in contact with skin and if swallowed
  S45 – In case of accident or if you feel unwell, seek medical advice immediately
  S36 – Wear suitable protective clothing
  S37 – Wear suitable gloves
- **Reagent K** – Histomount Mounting Solution contains toluene, which is highly flammable and harmful when inhaled. Refer to the MSDS for further information.
  R11 – Highly flammable
  R20 – Harmful by inhalation
  S2 – Keep out of the reach of children
  S16 – Keep away from sources of ignition – No smoking
  S25 – Avoid contact with eyes
  S29 – Do not empty into drains
  S33 – Take precautionary measures against static discharges
- All reagents designated as ready-to-use have been optimally diluted. Further dilution may adversely affect assay results.
- Incubation times, reagents and temperatures are optimized. Use of different incubation times, reagents or temperatures may adversely affect assay results.
- Use of different tissue fixatives or thicknesses is not recommended, and could adversely affect assay results.
- Consult local regulations for disposal of potentially toxic components.
- Minimize microbial contamination to avoid non-specific staining.
- Use ample precautions when handling reagents. Wear disposable gloves, coat, and safety glasses when handling suspected carcinogens.

Reagent storage

- Store SPOT-Light® HER2 CISH Kit should be stored at 2–8° C.
- Store **Reagent J** at room temperature (15–30°C).
  Note: Reagent B may be adversely affected if exposed to high temperature. Store this reagent at 2–8°C immediately after use. Do not store this reagent at room temperature.
- Store working PBS Tween 20 buffer at room temperature (15–30°C) for up to 1 week.

Do not use kit after expiration date printed on label. If product is stored under any conditions other than those specified in the package insert, then those storage conditions must be verified by the user. There are no visible signs that indicate the instability of this product; hence, it is important to evaluate the control slides. Please contact Technical Support if a problem with this kit is suspected.

Instructions for use

A. Specimen preparation

Paraffin-embedded tissue sections:

- Tissues fixed in neutral buffered formalin for 6–48 hours prior to paraffin embedding are suitable for use.
- Fixatives other than 10% neutral buffered formalin have not been optimized for this protocol and are not suitable for use.
- Tissue sections (4–5 µm thick) must be mounted on HistoGrip™-treated or Superfrost Plus microscope slides. Whenever possible, use tissue sections of standardized thickness to ensure uniform staining of CISH detection and counterstaining reagents [28]
- Air dry the slides, or dry at 37°C, and then bake for 2–4 hours at 60°C.
- Properly fixed and embedded tissues will keep indefinitely prior to sectioning and slide mounting if stored in a cool dry place (15–30°C).[26,27]
- Tissue sections 2–3 µm thick may give falsely low gene copy results.

B. Standard CISH procedure for FFPE tissue sections

Procedural notes

- All reagents except **Reagent C** (HER2 probe) should be equilibrated to room temperature (15–30°C) prior to use. The HER2 probe may be used cold without equilibrating to room temperature. Each incubation should be performed at room temperature (15–30°C), unless otherwise indicated.
- Throughout the entire procedure, unless otherwise indicated, it is important that the tissue section must not dry out between steps.
- Throughout the procedure multiple washes are required. For each wash, fresh solution must be used.
- The **Control Slides** are to be run together with each run of the patient test slides and must be treated in the same manner as the test specimens for all steps.
- Unless otherwise indicated, all steps are performed at room temperature (15–30°C).
- This procedure requires more than eight hours to complete: a convenient split is outlined below, starting on Day 1 with **Deparaffinization** through to **Denaturation and Hybridization** (overnight), and continuing on Day 2 with the **Stringent wash** through to **Brightfield microscopy**.

- Invitrogen reagents provided with the kit are necessary for the CISH procedure. Use of other reagents may result in high background, or a decrease or loss of CISH signal. Substitution of any of the reagents provided as kit components may nullify the results of the assay.

Day 1 procedure

1. **Reagent preparation**
   - **Xylene** (2 slide containers), **100% EtOH** (3 slide containers)
     - Cover tightly and store up to 1 week at room temperature (15–30°C) or until 100 slides have been processed.

   - **Ethyl alcohol (EtOH) series**
     - Prepare separate containers of 70%, 85%, 95% and 100% EtOH.
     - Cover tightly and store up to 1 week at room temperature (15–30°C) or until 100 slides have been processed.

     Label each wash reagent appropriately and note the order of use in the procedure, maintaining that order.

2. **Deparaffinization**

   Note: Prepare sufficient reagents for each slide container required for the number of slides in the run. Each wash requires a separate volume of reagent. If the next step cannot proceed immediately, air dry slides after soaking in 100% EtOH three times, instead of washing slides in dH_2O three times.

a)	Immerse in xylene	2 times, 5 min. each
b)	Soak in 100% EtOH	3 times, 3 min. each
c)	Wash in dH_2O	3 times, 2 min. each

   Complete deparaffinization is necessary for optimal and reproducible results.

3. **Heat pretreatment**

   Note: The slides must be boiled or heated at a temperature ≥98°C for 15 min. in Heat Pretreatment Solution (**Reagent A**). The slides should not be overheated by making sure that the temperature remains at 98°C to 100°C. We recommend that the hotplate be used for this step. (For protocols using a pressure cooker with a pressure and temperature gauge or microwave oven with temperature gauge, please contact Invitrogen Technical Support at tech_support@invitrogen.com). Heat Pretreatment Solution (**Reagent A**) can be used up to two times before discarding.

   a) Place slides in slide rack.
   b) Heat the Heat Pretreatment Solution (**Reagent A**) in a beaker on a hotplate until it is steadily boiling and temperature registers ≥ 98°C. To prevent buffer from evaporating, the beaker should be covered with either a glass cover or aluminum foil.
   c) Place slides in the boiling solution, cover the beaker, begin timing when the temperature reaches 98°C or when the boiling bubbles appear again, and boil for 15 min. Ensure that the temperature remains at 98°C to 100°C.
   d) Transfer slides immediately to dH_2O at room temperature (15–30°C).
   e) Wash three times in dH_2O, 2 min. each.

4. **Enzyme digestion**

   Note: For most breast tissues, 5 min. enzyme digestion at room temperature (15–30°C) will produce optimal CISH results. The digestion time should be adjusted based on the initial results with 5 min. digestion time. A different enzyme incubation time may be required, depending on tissue thickness and fixation method. An

enzyme digestion study indicated that enzyme digestion time can range from 2–20 min. and will yield suitable CISH signal for HER2 evaluation in a set of archival breast tissue samples. In a concordance study to support use of CISH vs. FISH,that used clinical breast cancer tissue specimens that had been processed within 12 months of the study date, the enzyme digestion time of 5 min. yielded optimal CISH signals for HER2 gene evaluation. [28]

a) Equilibrate the Enzyme Pretreatment Reagent (**Reagent B**) to room temperature (15–30°C).
b) Add enough **Reagent B** to cover the tissue section and incubate for 5 min. at room temperature (15–30°C).
c) Wash in dH_2O three times, 2 min. each.

5. **Dehydration in graded ethanol series:**
   a) 70% EtOH 2 min.
   b) 85% EtOH 2 min.
   c) 95 % EtOH 2 min.
   d) 100% EtOH 2 min.
   e) 100% EtOH 2 min.

6. **Air dry slides ≥20 min or until dry.** Label slides with pencil, if necessary.

7. **Denaturation and Hybridization**
   Note: Use a PCR thermal cycler with slide block or a heating block with digital temperature display and humidity slide chamber with 37°C incubator, or similar instrumentation. Ensure that the humidity of the chambers is adequately maintained. Hybridization performed for shorter time periods may result in a weaker signal. Probe denaturation at a temperature lower than recommended by the protocol may result in a weak or absent CISH signal. Changing the incubation times may result in either weak signal or background staining.

   a) Add 15 µl of HER2 probe (**Reagent C**) to the center of the 22 x 22 mm coverslip. Depending on tissue size, more or less probe may be required. Use the following probe volume based on coverslip size:
      - 18 mm x 18 mm 10 µl
      - 22 mm x 22 mm 15 µl
      - 24 mm x 30 mm 20 µl

   b) Place coverslip, probe side down, on the appropriate area of the tissue sample on slide. Invitrogen's CISH UnderCover™ Slips may be used in place of a standard coverslip. When using CISH UnderCover™ Slips, peel the paper backing off and place the side with the exposed coverslip (where the paper has just been removed) onto the appropriate area of the slide to cover the tissue sample. The edges of the tape should be pressed to seal the coverslip to prevent evaporation. DO NOT PRESS DOWN ON THE CENTER OF THE COVERSLIP.
   c) When using a standard coverslip, the coverslip must be sealed to prevent evaporation during incubation. Sealing can be accomplished using a 5 ml syringe and an 18G ½" needle. Fill the syringe with rubber cement and apply a thin layer to the edges of the coverslip, slightly overlapping onto the slide.
   d) Allow rubber cement to dry (~10 min.) to prevent coverslip from sliding off the slide.
   e) Denaturation and hybridization may be carried out using a PCR thermal cycler with slide block or a heating block with digital temperature display along with a humidity slide box and 37°C incubator.
      1) If using a PCR thermal cycler with slide block: Denature at 95°C (± 1°C) for 5 min., followed by overnight incubation (10–18 hrs) at 37°C (± 1°C).
      2) If using a heating block and humidity chamber with 37°C incubator: Denature at 95°C (± 1°C) for 5 min., followed by overnight incubation (10–18 hrs) at 37°C (± 1°C) in a humidity chamber.

Day 2 procedure

8. **Reagent preparation**

- **PBS (phosphate buffered saline)**
  - Add 1 pack of PBS powder (**Reagent E1**) to 1 L of dH_2O. Mix.
- **PBS/Tween 20 buffer (0.01% Tween)**
  - Add 10 drops 50% Tween 20 (**Reagent E2**) to 1 L of PBS (from above). Mix.
  - Store at room temperature (15–30°C) for up to 1 week.

- **Substrate-chromogen solution (DAB)**
  - Prepare this solution **immediately prior to use.**
  - Add 1 drop of each reagent (**I1, I2, I3**) to 1 mL dH_2O. Mix well.

- **3% H_2O_2 in absolute methanol**
  - Add 1 part of 30% H_2O_2 to 9 parts methanol.
  - Cover tightly and store up to 1 week at room temperature (15–30°C) or until 100 slides have been processed.

- **Xylene** (2 slide racks)
  - Cover tightly and store up to 1 week at room temperature (15–30°C) or until 100 slides have been processed.

9. Stringent wash

Note: Using temperatures higher than recommended by the procedure may produce a decrease or complete loss of the CISH signal. Washes at too low a temperature may result in high background. A calibrated thermometer must be used to ensure that the accurate temperature of the water bath is reached and maintained.

a) Turn on the 70°C (±1°C) water bath and bring it to temperature.
b) Prepare two Coplin jars containing SSC buffer (**Reagent D**), one at room temperature, the other heated to 70°C.
c) Peel off the rubber cement or UnderCover™ Slip. **Do not let the tissue section dry out.**
d) To remove coverslip without tearing tissue: Pre-soak the slides in room temperature SSC for ~2–3 min., until the coverslips slide off easily. Then proceed with the next step.
e) Rinse slides briefly in the jar containing room temperature (15–30°C) SSC, then immerse slides for 5 min. in the Coplin jar containing SSC in the 70°C (±1°C) water bath.
f) Wash slides in dH_2O 3 times, 2 min. each.

10. Immunodetection

a) Immerse slides in 3% H_2O_2 in 100% methanol 10 min.
b) Wash in PBS/Tween 20 (0.01%) 3 times, 2 min. each.
c) Add CAS-Block™ (**Reagent F**): 2–3 drops/slide or enough to cover tissue and incubate for 10 min. at room temperature (15–30°C).
d) Blot off **Reagent F** with a lab tissue. Do not rinse.
e) Add mouse anti-digoxigenin antibody (**Reagent G**): 2–3 drops/slide or enough to cover tissue and incubate for 30 min. at room temperature (15–30°C).
f) Wash in PBS/Tween 20 (0.01%) 3 times, 2 min. each.
g) Add goat anti-mouse HRP polymer conjugate (**Reagent H**): 2–3drops/slide or enough to cover tissue and incubate 30 min. at room temperature (15–30°C) in a humidity chamber.
h) Wash in PBS/Tween 20 (0.01%) 3 times, 2 min. each.
i) During the wash, prepare DAB substrate-chromogen solution: add one drop of each reagent (**Reagents I1, I2, I3**) to 1 ml of dH_2O.
j) Add substrate-chromogen solution (DAB): 2–3 drops/slide or enough to cover tissue and incubate for 30 min. at room temperature (15–30°C) in a humidity chamber.
k) Place slides in slide rack.
l) Wash with running tap water for 2 min.

11. Counterstaining and mounting

Note: Briefly counterstain for 3–5 sec. and examine the tissue under microscope without the coverslip. Counterstain for another 3–5 sec. if stronger nuclear staining is desired. Counterstaining time is dependent on tissues used. Dark counterstaining is not recommended, as it may obscure positive staining signals.

a) Counterstain tissue with hematoxylin (**Reagent J**): 3–5 sec.
b) Wash with running tap water for 2 min.
c) Dehydrate in graded EtOH series for 2 min. in each grade. (70%, 85%, 95%, 100%, 100%, saved from Day 1, and used in the same order.)
d) Immerse in xylene: 2 times, 2 min. each. This xylene wash **must be** different from that used on Day 1. It may be re-used in this step for 1 week, or up to 100 slides.
e) Coverslip using Histomount™ Mounting Solution (**Reagent K**).
f) Store slides at room temperature (15–30°C) for future analysis of results.

Limitations of the procedure

- The SPOT-Light® HER2 CISH Kit may not detect the reported 5%[10] of breast cancers that are positive by immmunohistochemistry (IHC) but negative for HER2 gene amplification.
- CISH is a multistep procedure that requires specialized training in the use of appropriate reagents, tissue selection, fixation, processing, preparation of the CISH slide, and interpretation of the staining results.
- Tissue staining is dependent upon the handling and processing of the tissue sample prior to staining. Excessive or incomplete counterstaining may compromise proper interpretation of results.
- Any deviation from the recommended test procedure may invalidate declared expected results; appropriate controls must be employed and documented. Users who deviate from the recommended test procedure must accept responsibility for interpretation of specimen results under those circumstances.
- The SPOT-Light® HER2 CISH Kit has been optimized only for identifying and quantifying the HER2/neu gene in interphase nuclei in formalin-fixed, paraffin-embedded human breast cancer tissue specimens. Other types of specimens or fixatives have not been validated.
- The clinical interpretation of any test results should be evaluated within the context of the patient's medical history and other diagnostic laboratory test results.

Quality control

Positive and negative controls on a single slide

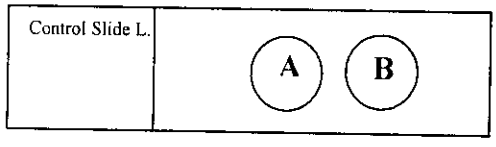

Image 1. Control Slide L contains non-amplified cell line A (MCF-7) and amplified cell line B (SK-OV-3). Note: cell lines are not drawn to scale, and are smaller than indicated in the diagram.

- CISH signal should be brown, distinct, and easy to evaluate.
- The included control slides (**Slide L**) contain two 4 µm sections cut from FFPE cell blocks prepared from two different cell lines. These slides should be used as procedural controls. **Cell line A (MCF-7)** is non-amplified and will have ≤5 signals or dots per nucleus. **Cell line B** (SK-OV-3) is amplified and will appear as small-to-large DAB clusters in the nucleus.
- The control cell line slide is to be run together with each run of the patient test slides and should be treated the same way as the tissue sections.
- If the control slide (**Slide L**) is negative for both A and B cell lines, this is an indication that an error occurred during the CISH procedure.
- The positive control cell line (B), known to demonstrate HER2 amplification, should be examined first to ascertain that all reagents are functioning properly. The presence of gene clusters, or >5 individual signals or dots, within the nucleus of a single cell is indicative of expected positive reactivity. If the positive control fails to demonstrate the presence of clusters or >5 signals per nucleus in a majority (>50%) of carcinoma cells, results obtained for the patient specimens should be considered invalid.

- The negative or normal control cell line (A), known to demonstrate HER2 non-amplification, should contain ≤5 dots in the nucleus of each cell.
- If nonspecific staining occurs in the nuclei of the normal control (A), results obtained for the patient specimens should be considered invalid.
- If nonspecific staining occurs, it usually exhibits a diffuse staining appearance. Occasionally, sporadic brown staining outside of nuclei may be observed in tissue sections that have been excessively formalin-fixed. Results in these cases should be interpreted carefully. Nonspecific staining should not be confused with positive CISH signals.
- A positive tissue control consisting of a breast cancer tissue known to demonstrate HER2 amplification, if used, should show the presence of gene clusters or >5 individual signals or dots per nucleus in a majority (>50%) of carcinoma cells, indicative of expected positive reactivity. If the positive tissue control fails to demonstrate the presence of clusters or >5 signals per nucleus in >50% of tumor cells, results obtained for the patient specimens should be considered invalid.
- A negative tissue control consisting of a breast cancer tissue known to demonstrate HER2 non-amplification, if used, should contain <5 signals per nucleus in a majority (>50%) of carcinoma cells.
- In general, the presence of no more than two signals or dots in the nuclei of the normal tissue (normal epithelial cells or stromal cells in the tumor) counterpart of the positive tissue control confirms that the probe and immunodetection reagents are not cross-reacting with cellular or tissue components. If nonspecific staining occurs in the nuclei of the normal tissue counterpart of the positive tissue control, results obtained for the patient specimens should be considered invalid.
- Variation in tissue processing and technical procedures in the user's laboratory must be validated as they may produce significant variability in results, necessitating regular performance of in-house controls in addition to the following procedures.

Interpretation

Assessing slide adequacy

The HER2 CISH dots (signals) should be small but clearly discernable using a 20X–40X objective. The dots will appear brown against the counterstain.

If there are no apparent dots on the sample, then the assay must be repeated. Please call Invitrogen Technical Support at 1-800-955-6288 (U.S. only) to discuss possible causes of the problem.

CISH signal appearance – refer to Appendix A, "HER2 CISH test interpretation guide"
HER2 CISH dots will appear as:

- A single dot (**Figure G**). A single dot has a smooth, rounded edge in normal or carcinoma cells. A single dot represents a single HER2 gene copy.
- A doublet (**Figure H**). Dots appear as "pairs" and do not represent a true small cluster. If two signals are separated by less than the diameter of one signal, these signals should be counted as a single dot.The doublet is the result of chromosomes dividing, with each signal residing on the pair of sister chromatids [29]
- A small cluster (**Figure E**). A small cluster is an irregularly shaped group of signals which is 3–5 times the diameter of a single dot. A single dot from the carcinoma or normal epithelial cells from the same slide can be used as a reference.
- A large cluster (**Figure A**). A large cluster is an irregularly shaped group of signals which is greater than 5 times the diameter of a single dot. A single dot from the carcinoma or normal epithelial cells from the same slide should be used as a reference.

Magnification	CISH signal
10X	Single dots are barely visible and easily overlooked.
20X	Single dots are small but clearly discernible.
40X	Single dots are easily identified.
60X or 100X	Not necessary.

Selecting the target fields for HER2 CISH signal enumeration:

- Using 4X–20X objectives, scan the CISH-stained specimen to identify the histopathologically most representative areas of invasive carcinoma. Avoid areas of necrosis, overlapping signals due to overlapping nuclei, and nuclei with weak signal intensity. Avoid intraductal carcinoma (DCIS) components in invasive carcinomas. Evaluate possible intratumoral heterogeneity of HER2 gene status before CISH enumeration.
- If the sample is homogenous, select an area of tissue with strong CISH signals for signal enumeration. Proceed to signal enumeration.
- If the sample has intratumoral heterogeneity of HER2 gene status in the invasive carcinoma component, select areas that represent each HER2 gene status for signal enumeration. A tissue sample is heterogeneous if the HER2 gene status (amplified and non-amplified cells) varies in different areas of the same section of a primary breast cancer. Cells in each area of heterogeneity need to be evaluated to determine which HER2 status (amplified or non-amplified) predominates in more than 50% of the tumor section. Proceed to signal enumeration of the area where the HER2 status is predominant for this tumor.

Signal enumeration

- Use a 40X objective. A higher objective may be used as necessary, but oil immersion is not necessary.
- Refer to **Appendix A**, "HER2 CISH test interpretation guide."
- An individual HER2 gene appears as a small round single dot (**Figure G**).
- Do not count nuclei that are overlapping or all areas in which nuclei are not visible. Count only the CISH signal that is inside a nucleus. Exclude occasional sporadic signals, which may be due to remnants of HER2 DNA spillage from the turnover of cancer cells, outside the nuclei.

<u>Non-amplification</u>

- Defined as 1–5 single dots in the majority (>50%) of carcinoma cells in the selected tissue area.
- It is not necessary to count the dots in 30 cells. Optional: use the worksheet in **Appendix B**, "HER2 CISH scoring scheme" for cell counting. Report results as the average of the total 30 cell count, to obtain a clear diagnosis of non-amplification.

<u>Amplification</u>

- Defined as:
  - >5 dots in the majority (>50%) of carcinoma cells in the selected tissue area, or
  - Large clusters in the majority (>50%) of carcinoma cells in the selected tissue area, or
  - A mixture of multiple dots and large clusters in the majority (>50%) of carcinoma cells in the selected tissue area, or
  - A mixture of multiple dots and small clusters in the majority (>50%) of carcinoma cells in the selected tissue area, or
  - Small clusters in the majority (>50%) of carcinoma cells in the selected tissue area.
- For any of the above cases, it is not necessary to count the dots in 30 cells. Optional: use the worksheet in **Appendix B**, "HER2 CISH scoring scheme," for cell counting. Report results as the average of the total 30 cell count to obtain a clear diagnosis of amplification. For counting purposes, count a small cluster as 5 dots, and a big cluster as 10 dots.
  - Rationale: The assignment of specific dot numbers to small or large clusters is arbitrary for quantification purposes. Cells normally have two HER2 gene copies. Cells that have replicated their DNA but have not yet divided have 4 gene copies. Hence, a small cluster is an indication of amplification and will have a minimum number of 5 gene copies. A large

cluster, at least double the size of a small cluster, will have a minimum number of 10 gene copies.

- **Unclear cases of amplification or non-amplification**

  Interpret specimens that do not clearly belong to amplification or non- amplification categories with care. These specimens exhibit 4–6 dots in the majority (>50%) of carcinoma cells in the selected target field. When the average number of dots is between 4 and 6 after 30 carcinoma cells have been counted, an additional 30 cells should be counted for a total of 60 cells.

  If still in doubt, the assay should be repeated with a fresh specimen slide. Report results using the worksheet in **Appendix B**, "HER2 CISH scoring scheme."

  1. Count the dots in 30 tumor cells in the selected tissue area. Refer to #4 below if small clusters are evident.
  2. Total the number of dots in the 30 cells and calculate the average.
  3. Record the result based on the average of 60 cells
  4. If a mixture of single dots and small clusters (due to the clustering of several single dots) is observed, or small clusters only are observed:
     - Count one small cluster as 5 dots.
     - A single dot from the carcinoma or normal epithelial cells from the same slide can be used as a reference in determining what represents a small cluster.
  5. Report results as the average of the total 60 cell count to obtain a diagnosis of either amplification or non-amplification under the Invitrogen Guideline.".

- **Reporting of results**

  Results may be reported using the worksheet in Appendix B, "HER2 CISH scoring scheme." The HER2 status report must be recorded per the Invitrogen Guideline, especially for unclear cases of amplification or non-amplification.

Interpretation summary

Amplification	>5 dots, or large clusters, or mixture of multiple dots and large clusters, or mixture of multiple dots and small clusters, or small clusters of the HER2 gene present per nucleus in a majority (>50%) carcinoma cells in the tissue area selected for enumeration. See **Figures A, B, C, D and E** in **Appendix A, "HER2 CISH test interpretation guide**." A large cluster is an irregularly shaped group of signals, which is greater than 5 times the diameter of a single dot. A single dot from the carcinoma or normal epithelial cells from the same slide must be used as a reference. See **Appendix A, Figure A**. A small cluster is an irregularly shaped group of signals, which is 3–5 times the diameter of a single dot. A single dot from the carcinoma or normal epithelial cells from the same slide must be used as a reference. See **Appendix A, Figure E**.
Non-amplification	1–5 dots of the HER2 gene present per nucleus in a majority (>50%) of carcinoma cells in the tissue area selected for enumeration. See **Appendix A, Figure F, Figure G, and Figure H.** A single dot has a smooth, rounded edge and is present in normal and carcinoma cells.

Expected values

The prevalence of amplified HER2 gene in the general population of women with breast cancer is 18–30%.[8, 9] When comparing HER2 gene amplification to HER2 protein overexpression, studies have shown that up to 5% of breast cancer patients are positive for protein overexpression when tested by immunohistochemistry (IHC) but negative for HER2 gene amplification.[10]

Specific performance characteristics

Clinical performance

The safety and effectiveness of the SPOT-Light® HER2 CISH Kit were evaluated in a comparative study of three tests: the SPOT-Light® HER2 CISH Kit, PathVysion® FISH, and HercepTest™. The study involved two sets of cases: 226 consecutive cases and 60 supplemental cases. The consecutive cases were selected from consecutive invasive breast cancer cases examined for patient care at two study sites (sites A & B). The supplemental cases were cases that had been scored 2+ by immunohistochemistry (IHC) (using antibody AB8) during patient care at site A.

A. Consecutive cases

The following table summarizes the distribution of CISH and FISH results in relation to HercepTest™ scores. A test was considered valid when the resulting stained slide was acceptable for evaluation.

Table 1: Results of IHC, FISH, and CISH

Protein expression, HercepTest™ Score		0	1	2	3	Total
IHC cases	(N)	141	19	21	40	221
	(%)[1]	63.8%	8.6%	9.5%	18.1%	100%
Gene ratio with FISH HER2 status						
Number of valid FISH cases[2]		140	19	21	38	218
Amplified n (%)[3]		1 (0.5)	0 (0.0)	5 (2.3)	31 (14.2)	37
Non-amplified n (%)[3]		139 (63.8)	19 (8.7)	16 (7.3)	7 (3.2)	181
Gene copies with CISH HER2 status						
Number of valid CISH cases[2]		132	17	19	38	206
Amplified n (%)[3]		1 (0.5)	0 (0.0)	3 (1.5)	32 (15.5)	36
Non-amplified n (%)[3]		131 (63.6)	17 (8.3)	16 (7.8)	6 (2.9)	170

[1] % = N ÷ Total N × 100%

[2] Number of valid FISH (or CISH) cases with the corresponding IHC score.

[3] % = n ÷ Total number of valid FISH (or CISH) cases × 100%

Table 2: Agreement between CISH and IHC

CISH results	IHC results		Total
	Positive (3+)	Negative (<3+)	
Amplified	32	4	36
Non-amplified	6	164	170
Total	38	168	206

20 cases were reported with either missing or invalid IHC or CISH test outcomes and were excluded from the table.

Positive agreement	=	**32/38**	**= 84.2%**	**(95% CI: 68.8%, 94.0%)**
Negative agreement	=	**164/168**	**= 97.6%**	**(95% CI: 94.0%, 99.4%)**
Total percentage agreement	=	**(32+164)/206**	**= 95.1%**	**(95% CI: 91.3%, 97.7%)**

The results showed a concordance of 95.1% (95%CI: 91.3% –97.7%), indicating strong agreement between the SPOT-Light® HER2 CISH Kit and HerceptTest™.

Table 3: Agreement between CISH and FISH

CISH results	FISH results		Total
	Amplified	Non-amplified	
Amplified	34	0	34
Non-amplified	2	169	171
Total	36	169	205

21 cases were reported with either missing or invalid FISH or CISH test outcomes and were excluded from the table.

Positive agreement	=	**34/36**	**= 94.4%**	**(95% CI: 81.3%, 99.3%)**
Negative agreement	=	**169/169**	**= 100.0%**	**(95% CI: 97.8%, 100.0%)**
Total percentage agreement	=	**(34+169)/205**	**= 99.0%**	**(95% CI: 96.5%, 99.9%)**

The results showed a concordance of 99.0% (95% CI: 96.5%–99.9%), indicating strong agreement between the SPOT-Light® HER2 CISH Kit test and the PathVysion® HER2 test. Thus, the agreement rate indicated that the SPOT-Light® HER2 CISH Kit test gave results that were equivalent to the PathVysion® HER2 test.

A summary of the 2 discordant cases between the SPOT-Light® HER2 probe test and PathVysion® HER2 probe test is shown below. CISH and FISH data are presented as mean and range, and the IHC column shows the HercepTest™ score.

Table 4: Discordant cases between FISH and CISH

HER2 CISH (Pos)/FISH (Neg)				HER2 CISH (Neg)/FISH (Pos)			
Case number	CISH	FISH	IHC	Case number	CISH	FISH	IHC
(2, 0.98%)[1]							
				A-095-01	4.07 (1.00 – 10.00)	2.81 (1.67 – 5.00)	2+
				B-008	2.77 (2.00 – 4.00)	2.65 (1.00 – 6.00)	2+

[1]% = number of discordant cases divided by total number of cases with valid FISH and CISH from the corresponding site × 100%

B. Supplemental IHC 2+ cases

An additional 60 IHC 2+ supplemental cases were provided by study site A and tested at study sites A and B. The results at each study site demonstrated that the SPOT-Light® HER2 CISH Kit test gave results that were equivalent to the PathVysion® HER2 test. The correlation results are summarized in Tables 5 and 6.

Table: 5 Agreement between CISH and FISH at study site A on IHC 2+ Cases

CISH results	FISH results		Total
	Amplified	Non-amplified	
Amplified	6	0	6
Non-amplified	2	46	48
Total	8	46	54

6 cases were reported with either missing or invalid

Positive agreement	=	**6/8**	**= 75.0%**	**(95% CI: 34.9%, 96.8%)**
Negative agreement	=	**46/46**	**= 100.0%**	**(95% CI: 92.3%, 100.0%)**
Total percentage agreement	=	**(6+46)/54**	**= 96.3%**	**(95% CI: 87.3%, 99.6%)**

Table 6: Agreement between CISH and FISH at study site B on IHC 2+ Cases

CISH results	FISH results		Total
	Amplified	Non-amplified	
Amplified	7	2	9
Non-amplified	2	45	47
Total	9	47	56

4 cases were reported with either missing or invalid

Positive agreement	=	**7/9**	**= 77.8%**	**(95% CI: 40.0%, 97.2%)**
Negative agreement	=	**45/47**	**= 95.7%**	**(95% CI: 85.5%, 99.5%)**
Total percentage agreement	=	**(7+45)/56**	**= 92.9%**	**(95% CI: 82.7%, 98.0%)**

Table 7: Discordant IHC 2+ cases between CISH and FISH

HER2 CISH (Pos)/FISH (Neg)				HER2 CISH (Neg)/FISH (Pos)			
Case number	CISH	FISH	IHC	Case number	CISH	FISH	IHC
Site A (2, 3.70%)[1]							
				S-156-01	2.77 (1.00 – 5.00)	2.48 (0.50 – 5.00)	2+
				S-173-01	3.67 (1.00 – 7.00)	2.34 (0.50 – 8.00)	2+
Site B (4, 7.14%)[1]							
S-171	5.20 (2.00 – 8.00)	1.08 (0.50 – 2.00)	3+	S-156	5.00 (1.00 – 9.00)	2.23 (1.00 – 5.00)	3+
S-178	20.00 (20.00 – 20.00)	1.25 (0.50 – 2.00)	0	S-183	4.13 (3.00 – 6.00)	2.73 (1.00 – 6.00)	0

[1] % = number of discordant cases divided by total number of cases with valid FISH and CISH from the corresponding site × 100%

C. **HercepTest 2+ cases**

All consecutive and supplemental cases were tested with HercepTest™. Cases that yielded HercepTest™ 2+ scores were combined and tested at both Study Sites. The results at each study site demonstrated that the SPOT-Light® HER2 CISH Kit gave results that were equivalent to the PathVysion® HER2 test. The correlation results are summarized in Tables 8 & 9.

Table 8: Agreement between CISH and FISH on HercepTest 2+ Cases at study site A

CISH results	FISH results		Total
	Amplified	Non-amplified	
Amplified	3	0	3
Non-amplified	4	27	31
Total	7	27	34

2 cases were reported with either missing or invalid FISH or CISH test outcomes and were excluded from the table.

Positive agreement	=	**3/7**	**= 42.9%**	**(95% CI: 9.9%, 81.6%)**
Negative agreement	=	**27/27**	**= 100.0%**	**(95% CI: 87.2%, 100.0%)**
Total percentage agreement	=	**(3+27)/34**	**= 88.2%**	**(95% CI: 72.6%, 96.7%)**

Table 9: Agreement between CISH and FISH on HercepTest 2+ Cases at study site B

CISH results	FISH results		Total
	Amplified	Non-amplified	
Amplified	4	1	5
Non-amplified	1	35	36
Total	5	36	41

2 cases were reported with either missing or invalid FISH or CISH test outcomes and were excluded from the table.

Positive agreement	=	**4/5**	**= 80.0%**	**(95% CI: 28.4%, 99.5%)**
Negative agreement	=	**35/36**	**= 97.2%**	**(95% CI: 85.5%, 99.9%)**
Total percentage agreement	=	**(4+35)/41**	**= 95.1%**	**(95% CI: 83.5%, 99.4%)**

Table 10: HercepTest 2+-discordant cases between CISH and FISH

HER2 CISH (Pos)/FISH (Neg)				HER2 CISH (Neg)/FISH (Pos)			
Case number	CISH	FISH	IHC	Case number	CISH	FISH	IHC
Site A (4, 11.76%)[1]							
				A-095-01	4.07 (1.00 – 10.00)	2.81 (1.67 – 5.00)	2+
				B-008-08	1.77 (1.00 – 4.00)	2.45 (1.00 – 5.00)	2+
				S-156-01	2.77 (1.00 – 5.00)	2.48 (0.50 – 5.00)	2+
				S-173-01	3.67 (1.00 – 7.00)	2.34 (0.50 – 8.00)	2+
Site B (2, 4.88%)[1]							
A-023	5.20 (2.00 – 8.00)	1.49 (0.67 – 3.50)	2+	B-008	2.77 (2.00 – 4.00)	2.65 (1.00 – 6.00)	2+

[1] % = number of discordant cases divided by total number of cases with valid FISH and CISH from the corresponding site × 100%

D. Polysomy cases

Two study sites evaluated the polysomy cases based on their respective institutional clinical practices. At site A, polysomy of chromosome 17 was defined as the presence of ≥3 CEP17 signals in at least 10% of the tumor cells, whereas at site B the criteria was the presence of ≥3 CEP17 signals in at least 30% of the tumor cells. The frequency of tumors with polysomy of chromosome 17 was 18.68% at site A and 7.91% at site B. The detection rate of polysomy on the same tumor set varied substantially between the two test sites due to the difference in the definition of polysomy used at each site. The agreement level obtained independently at each study site, indicated that the SPOT-Light® HER2 CISH Kit gave results that were equivalent to the PathVysion® HER2 test. The results are summarized in Tables 11 and 12.

Table 11: Agreement of CISH and FISH on Polysomy Cases at study site A

CISH results	FISH results		Total
	Amplified	Non-amplified	
Amplified	12	0	12
Non-amplified	2	34	36
Total	14	34	48

3 cases were reported with either missing or invalid FISH or CISH test outcomes and were excluded from the table.

Positive agreement	=	**12/14**	**= 85.7%**	**(95% CI: 57.2%, 98.2%)**
Negative agreement	=	**34/34**	**= 100.0%**	**(95% CI: 89.7%, 100.0%)**
Total percentage agreement	=	**(12+34)/48**	**= 95.8%**	**(95% CI: 85.8%, 99.5%)**

Table 12: Agreement of CISH and FISH on Polysomy Cases at study site B

CISH results	FISH results		Total
	Amplified	Non-amplified	
Amplified	12	1	13
Non-amplified	0	9	9
Total	12	10	22

0 cases were reported with either missing or invalid FISH or CISH test outcomes and were excluded from the table.

Positive agreement	=	**12/12**	**= 100.0%**	**(95% CI: 73.5%, 100.0%)**
Negative agreement	=	**9/10**	**= 90.0%**	**(95% CI: 55.5%, 99.8%)**
Total percentage agreement	=	**(12+9)/22**	**= 95.5%**	**(95% CI: 77.2%, 99.9%)**

Table 13: Discordant Polysomy cases between CISH and FISH

HER2 CISH (Pos)/FISH (Neg)				HER2 CISH (Neg)/FISH (Pos)			
Case number	CISH	FISH	IHC	Case number	CISH	FISH	IHC
Site A (2, 4.17%)[1]							
				A-095-01	4.07 (1.00 – 10.00)	2.81 (1.67 – 5.00)	2+
				S-156-01	2.77 (1.00 – 5.00)	2.48 (0.50 – 5.00)	2+
Site B (1, 4.55%)[1]							
A-023	5.20 (2.00 – 8.00)	1.49 (0.67 – 3.50)	2+				

[1] % = number of discordant cases divided by total number of cases with valid FISH and CISH from the corresponding site × 100%

E. Summary of comparison results between the SPOT-Light® HER2 CISH Kit and PathVysion® HER2 DNA Probe Kit

Table 14: Summary of SPOT-Light® HER2 CISH Kit and PathVysion® HER2 DNA Probe Kit

Type of cases	Consecutive cases	Supplemental cases		Equivocal cases		Polysomy cases	
		Site A	Site B	Site A	Site B	Site A	Site B
Number of samples	205	54	56	34	41	48	22
Positive % agreement	94.4%	75.0%	77.8%	42.9%	80.0%	85.7%	100.0%
Negative % agreement	100.0%	100.0%	95.7%	100.0%	97.2%	100.0%	90.0%
Total % agreement	99.0%	96.3%	92.9%	88.2%	95.1%	95.8%	95.5%

Analytical sensitivity

The sensitivity of the SPOT-Light® HER2 CISH Kit was tested using FFPE breast cancer tissue sections with non-amplified and amplified HER2 status, as well as the FFPE cell lines used in the control slides. The HER2 gene was detected as a single dot on the tissue section and cell block section with normal HER2 gene. Each amplified and non-amplified sample demonstrated 3+ intensity staining and good tissue morphology.

Hybridization efficiency

The hybridization efficiency was established by testing 2 tissue samples (1 amplified, 1 non-amplified, 10 sections each) and 4 cell line samples (2 amplified, 2 non-amplified). Each sample was analyzed for the number of cells with HER2 signals out of the total number of cells analyzed (100–300 cells). The hybridization efficiency was established to be 94.7–100%. For CISH staining on 226 clinical specimens from 3 laboratories, the failure rate at each site was 8.4% (19), 1.3% (3), and 0.9% (2).[28]

Analytical specificity

To determine specificity, metaphase studies were performed on cytogenetically prepared slides, PCR was performed using a HER2 gene-specific primer pair on the HER2 DNA probe template, and DNA sequencing was performed for both ends of the BAC clones used in the HER2 DNA probe.

The SPOT-Light® CISH HER2 probe was demonstrated to bind specifically to the HER2 gene locus on chromosome band 17q11.2-21. Chromosome localization was established by metaphase FISH in normal lymphocytes; PCR testing demonstrated the desired DNA band, and DNA sequencing demonstrated the correct chromosome location and coverage of the HER2 gene.

Procedural limits
The SPOT-Light® HER2 CISH procedure was tested at the extremes of each of the following parameters: tissue thickness, pretreatment, denaturation, hybridization, stringent wash, immunodetection, and counterstaining. No significant differences in CISH results were observed under the following conditions:

Table 15: Procedural limits

	Assay parameter acceptable ranges		
Tissue thickness		4–6 μm	
Heat pretreatment		99–100°C	10–20 min
Enzyme digestion			4-14 min
Denaturation	PCR thermal cycler	93–98°C	2–-8 min
Hybridization		30–39° C	10–18 h
Stringent wash		60–78° C	2–8 min
Immunodetection	HRP polymer conjugate		25v60 min
	DAB chromogen		15–60 min
Counterstaining			3–30 sec.

Reproducibility
Three separate lots of the SPOT-Light® HER2 CISH Kit were tested on 3 breast cancer tissue samples and 4 cell line samples, with different levels of HER2 gene. There was no change in performance of the assay across the 3 lots tested.

Inter-run (day-to-day) reproducibility
The study performed CISH inter-run reproducibility on three different days using breast cancer specimens with three types of HER2 status with 3 specimens of each type. The summary is as follows:

Table 16: Inter-run (day-to-day) reproducibility

Gene copy	N	Non-amplified	Non-amplified, polysomy	Amplified
Average N =	**9**	**1.79**	**3.45**	**20.22**
Std dev.	**9**	**0.05**	**0.12**	**1.72**
CV	**9**	**3%**	**4%**	**8%**

Observer-to-observer study
Three pathologists were trained to read slides prepared and stained using the SPOT-Light® HER2 CISH Kit. To validate proficiency, each pathologist was given eight prestained slides (6 non-amplified and 2 amplified) provided by Invitrogen. With both types of cases (non-amplified and amplified) all samples (100%) were interpreted accurately.

Table 17: Observer-to-observer reproducibility

Slide ID	Slide #	CISH Signal, Average HER2 CISH dots/cell, and HER2 Gene Status		
		Observer 1	Observer 2	Observer 3
1	Sample 1 (NAM)	2 dots NAM	1-5 dots NAM	1-2 NAM
2	Sample 2 (AM)	Large Cluster + multiple dots AM	Large Cluster + dots AM	>10 AM
3	Sample 3 (NAM)	2-5 NAM	1-5 NAM	3-5 NAM
4	Sample 4 (NAM)	2-4 NAM	2.8 NAM	3-5 NAM
5	Sample 5 (NAM)	2 NAM	1-5 NAM	1-2 NAM
6	Sample 6 (NAM)	2-5 NAM	1-5 NAM	4 NAM
7	Sample 7 (AM)	Large cluster + multiple dots AM	Large cluster AM	Large cluster + multiple dots AM
8	Sample 8 (NAM)	2-5 NAM	3.4 NAM	4.3 NAM

Site-to-site reproducibility

The site-to-site CISH reproducibility study was based on 226 consecutive cases repeated at three study sites. Total agreement percentages for CISH reproducibility using >5 as the cutoff for positivity were 99.0%, 98.6%, and 98.1%, indicating strong reproducibility for testing with the SPOT-Light® HER2 CISH Kit.

Table 18: CISH Reproducibility for All Consecutive Cases Examined by Site A and B

Site A CISH Results	Site B: CISH Results		Total
	Amplified	Non-amplified	
Amplified	34	0	34
Non-amplified	2	170	172
Total	36	170	206

20 cases were reported with either missing or invalid CISH test outcomes and were excluded from the table.

Total percentage agreement = (34+170)/206 99.0% (95% CI: 96.5%, 99.9%)

Table 19: CISH Reproducibility for All Consecutive Cases Examined by Site C and Site B

Site C CISH Results	Site B: CISH Results		Total
	Amplified	Non-amplified	
Amplified	35	0	35
Non-amplified	3	184	187
Total	38	184	222

4 cases were reported with either missing or invalid CISH test outcomes and were excluded from the table.

Total percentage agreement = (35+184)/222 98.6% (95% CI: 96.1%, 99.7%)

Table 20: CISH Reproducibility for All Consecutive Cases Examined by Site C and Site A

Site C CISH Results	Site A: CISH Results		Total
	Amplified	Non-amplified	
Amplified	32	1	33
Non-amplified	3	171	174
Total	35	172	207

19 cases were reported with either missing or invalid CISH test outcomes and were excluded from the table.

Total percentage agreement = (32+171)/207 98.1% (95% CI: 95.1%, 99.5%)

Repeatability and Reproducibility Studies on Consecutive Tissue Sections and Various Tissue Thickness

The repeatability and reproducibility studies were conducted to evaluate the SPOT-Light® HER2 CISH™ Kit repeatability and reproducibility when testing consecutive non-amplified and amplified breast cancer tissues of varying thicknesses.

The samples under evaluation included slides from three breast cancer tissue blocks: HER2 non-amplification (normal), HER2 borderline amplification, and HER2 amplification (high). Ten samples per block of consecutive sections of 4 µm thickness were processed and tested according to standard procedures (control condition), and samples of different thicknesses (range 2-8 µm) from each block were similarly processed and tested in duplicate according to standard procedures. The results from this testing appear in Tables 21-23.

Table 21. Average HER2 CISH dots per cell in consecutive sections at 4 µm (breast cancer with *normal* HER2)

	Section Number									
	1	2	3	4	5	6	7	8	9	10
Average HER2 CISH dots/nucleus	1.8	2.0	1.9	2.0	1.6	1.7	1.7	1.6	1.7	2.0
Average HER2	1.8									
STD	0.16									
%CV	8.9									

Table 22. Average HER2 CISH dots per cell in consecutive sections at 4 µm (breast cancer with *borderline amplification* of HER2)

	Section Number									
	1	2	3	4	5	6	7	8	9	10
Average HER2 CISH dots/nucleus	5.4	5.5	5.3	5.8	5.2	6.2	5.7	5.4	5.5	5.8

Average HER2	5.6
STD	0.30
%CV	5.4

Table 23. Average HER2 CISH dots per cell in consecutive sections at 4 µm (breast cancer with *amplified* HER2)

	Section Number									
	1	2	3	4	5	6	7	8	9	10
Average HER2 CISH dots/nucleus	BC	BC	BC	BC	BC	BC	BC	BC	BC	BC

Troubleshooting

Problem	Possible cause	Suggested action
Weak or no signal	1. Pretreatment instructions were not followed.	1. Ensure that correct pretreatment conditions were used. Refer to the procedure.
	a) Incorrect heat pretreatment conditions (incubation time or temperature)	a) Ensure that correct heat pretreatment conditions were used: 15 minutes at 98–102°C.
	b) Incorrect enzyme pretreatment conditions (time or temperature)	b) Ensure that correct enzyme pretreatment conditions were used (5 min. recommended, 2–20 min. range). Ensure that the enzyme is brought to room temperature (15–30°C) prior to use.
	c) Tissue section thicker than optimal	c) Depending on tissue thickness (4–6 µm) and fixation conditions, optimal enzyme pretreatment incubation time may need to be increased or decreased.
	2. Incorrect denaturation conditions	2. Ensure that correct denaturation conditions were used. Refer to the procedure.
	a) Incorrect denaturation temperature	a) Ensure denaturation temperature is 95°C (range of 95–98°C) for PCR thermal cycler and heating block, 90°C (range of 85–90°C)
	b) Incorrect denaturation time	b) Ensure denaturation time is 5 min. for PCR thermal cycler,.
	3. Incorrect stringent wash conditions	3. Ensure that correct stringent wash conditions were used. Refer to the procedure.
	a) Incorrect stringent wash temperature (exceeds 80°C)	a) Ensure stringent wash is carried out at 70°C. A calibrated thermometer is recommended to verify temperature.
	b) Incorrect stringent wash incubation time	b) Ensure correct stringent wash incubation time (5 min.)
	4. Excessively high temperature during storage or transport	4. Check storage conditions. Reagents B, F–I, K, and L should be stored at 2–8°C; Reagent C should be stored at –20°C.
Poor tissue morphology	1.Incorrect heat pretreatment conditions (time or temperature)	1. Ensure that correct heat pretreatment conditions were used.
	2. Incorrect enzyme pretreatment conditions (time or temperature)	2. Ensure that correct enzyme digestion conditions were used. For most breast cancer tissues, 5 min. at room temperature (15–30°C) is optimal. Depending on tissue thickness and fixation time, optimal digestion incubation time may need to be increased or decreased.
	3. Incorrect denaturation conditions (time or	3. Ensure that correct denaturation conditions were used. Refer to the procedure.

Problem	Possible cause	Suggested action
	temperature) 4. Coverslip removal caused physical damage to the tissue 5. Tissue allowed to dry during CISH procedure 6. Incorrect tissue thickness 7. Incorrect fixation time 8. Incorrect fixative	4. Do not press down on the coverslip. Pre-soak the slides in room temperature (15–30 °C) SSC buffer for 2–3 min. or until the coverslip slides off easily. 5. Unless specifically stated, do not let tissue dry at any time during CISH procedure. 6. Ensure tissue is 4–6 µm. 7. Ensure tissue is fixed for 6–48 hours. 8. Ensure that tissue is fixed in 10% neutral buffered formalin.
Background staining	1. Incorrect stringent wash temperature (<70°C) 2. Excessive incubation with DAB 3. Incorrect reagents used as wash buffer	1. Ensure correct stringent wash temperature (70°C). 2. Ensure correct DAB incubation time is 30 min. at room temperature (15–30°C). 3. Wash buffer (**Reagents E1 + E2**) must be used during the immunodetection steps.
Signals apparent but difficult to see	1. Excessive hematoxylin counterstaining	1. Ensure tissue is counterstained for 3–5 seconds. If uncertain of optimal staining time, counterstain tissue for 3 sec., then wash for 2 min. under tap water. Check counterstained tissue under microscope. If still too light, counterstain for another 3–5 sec. before proceeding to coverslipping.
Areas without signal	1. Air bubbles formed under coverslip upon addition of probe 2. Insufficient probe volume for tissue size	1. Ensure that air bubbles did not form when adding probe and coverslip. 2. Add ~15 µl for tissue that is adequately covered by a 22 x 22 mm coverslip. More probe and a larger coverslip may be required for larger tissues (use 20 µl for a tissue using a 24 x 30 mm coverslip.)

NOTE: If problem is not listed above or if suggested action does not resolve the issue, please call Technical Support at 1-800-955-6288, (US only) or send an e-mail to tech_support@invitrogen.com.

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TRADEMARKS

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URL: www.invitrogen.com/pathology

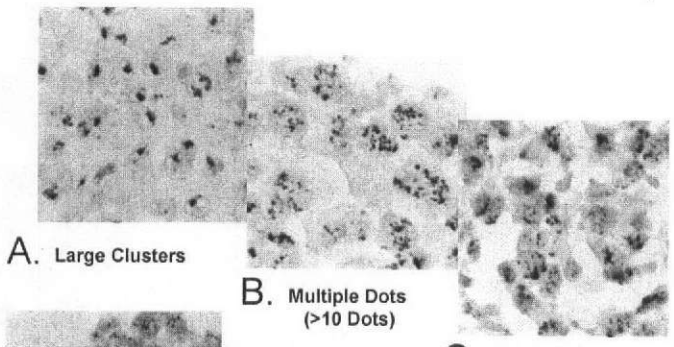
Explanation of symbols

REF	Catalog number	LOT	Lot number
	Temperature limitation		Manufacturer
	Contains sufficient for <n> tests	EC REP	EC Authorized Representative
	Use by	IVD	*In vitro* diagnostic
	Consult instructions for use		Consult accompanying documents

EC REP for IVDD 98/79/EC: Invitrogen Ltd., Inchinnan Business Park, 3 Fountain Drive, Paisley, PA4 9RF, UK

Appendix A: HER2 CISH test interpretation guide

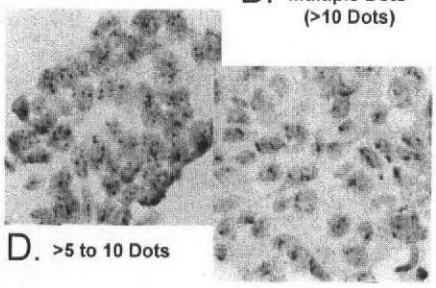
HER2 gene amplification



A. Large Clusters

B. Multiple Dots (>10 Dots)

C. Large Clusters And Multiple Dots



D. >5 to 10 Dots

E. Small Clusters

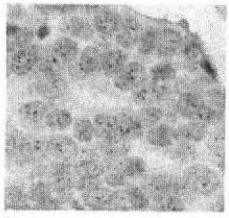
Amplification
High amplification: >10 dots, or large clusters, or a mixture of multiple dots and large clusters of the HER2 gene present per nucleus in >50% cancer cells. See Figures A, B, and C.

A large cluster is an irregularly shaped group of signals, which is greater than 5 times the diameter of a single dot. A single dot from the tumor or normal epithelial cells from the same slide must be used as a reference. See Figure A.

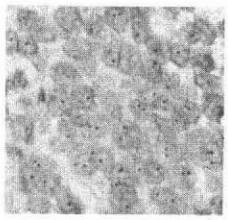
Low amplification: >5 dots to 10 dots, or small clusters, or mixture of multiple dots and small clusters of the HER2 gene present per nucleus in >50% cancer cells. See Figures D and E.

A small cluster is an irregularly shaped group of signals, which is 3–5 times the diameter of a single dot. A single dot from the tumor or normal epithelial cells from the same slide must be used as a reference. See Figure E.

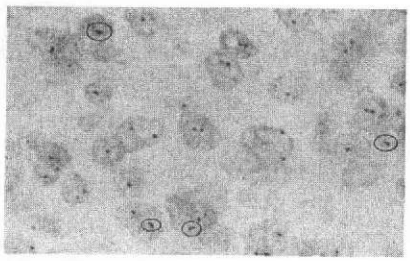
HER2 gene non-amplification



F. Polysomy: 3 - 5 Dots



G. Diploid: 1- 2 Dots



H. Doublet = 1 Dot

Non-amplification
Polysomy: 3–5 dots of the HER2 gene present per nucleus in >50% of cancer cells. See Figure F.
A single dot has a smooth, rounded edge in normal or tumor cells. See Figure F.

Diploid: 1–2 dots of the HER2 gene present per nucleus in >50% of cancer cells. See Figure G.
A single dot has a smooth, rounded edge in normal or tumor cells. See Figure G.

Doublet
If two signals are separated by less than the diameter of a single signal, these signals should be counted as a single dot. Doublets appear as a pair and do not represent true small clusters. (Image courtesy of Dr. Adrienne Morey)

Appendix B: HER2 CISH scoring scheme
SPOT-Light® HER2 CISH Kit, Cat. No. 84-0150

Staining Run Log ID:_____

SPOT-Light® HER2 CISH Kit. 84-0150 Lot:_____ Specimen ID:_____

CISH result: (add the number of HER2 signals and the number of cells evaluated in the appropriate line):

Chart 1 (CISH)

Cell #	Dots of HER2 or clusters	Cell #	Dots of HER2 or clusters
1		16	
2		17	
3		18	
4		19	
5		20	
6		21	
7		22	
8		23	
9		24	
10		25	
11		26	
12		27	
13		28	
14		29	
15		30	

Note: If clusters, please provide best estimate of dots/gene copies (small cluster = 5 dots, big cluster = 10 dots).

Sum of dots in 30 cells _____
Average of dots in 30 cells _____

If the average is between 4 and 6 dots, count dots in another 30 cells for a total of 60 cells and record results in the Chart 2. If average is <4 or >6, it is not necessary to count another 30 cells and the sample may be recorded as amplification or non-amplification.

Date and Signature, Technician:_____

Date and Signature, Pathologist:_____

Chart 2 (CISH). If needed, enter the number of dots in the second set of 30 cells:

Cell #	Dots of HER2 or clusters	Cell #	Dots of HER2 or clusters
31		46	
32		47	
33		48	
34		49	
35		50	
36		51	
37		52	
38		53	
39		54	
40		55	
41		56	
42		57	
43		58	
44		59	
45		60	

Sum of dots in cells 31-60 _____
Sum of dots in cells 1-30 _____
Average of dots of 60 cells _____

Invitrogen has not validated the use of the cutoffs and/or the interpretation recommendations from the ASCO/CAP 2007 practice guidelines.[25]

CISH Scoring

Invitrogen HER2 scoring guideline	Record results:
• Non-amplification: 1–5 signals/nucleus in tumor cells • Amplification: >5 signals/nucleus, or cluster of amplified signals/nucleus in >50% of tumor cells	Non-amplification (≤5) _____ Amplification (>5) _____

Date and Signature, Technician: _____

Date and Signature, Pathologist: _____